Published in final edited form as:

J Am Chem Soc. 2017 May 24; 139(20): 7110–7116. doi:10.1021/jacs.7b03395.

LNA Thymidine Monomer Enables Differentiation of the Four Single-Nucleotide Variants by Melting Temperature

Judy M. Obliosca[†], Sara Y. Cheng[‡], Yu-An Chen[†], Mariana F. Llanos[§], Yen-Liang Liu[†], Darren M. Imphean[†], David Bell[†], Jeffrey T. Petty[§], Pengyu Ren[†], and Hsin-Chih Yeh^{†,*}

[†]Department of Biomedical Engineering, Cockrell School of Engineering, University of Texas at Austin, Austin, TX 78712, USA

[‡]Department of Physics, College of Natural Sciences, University of Texas at Austin, Austin, TX 78712, USA

[§]Department of Chemistry, Department of Chemistry, Furman University, Greenville, South Carolina 29613, USA

Abstract

High-resolution melting (HRM) analysis of DNA is a closed-tube single-nucleotide polymorphism (SNP) detection method that has shown many advantages in point-of-care diagnostics and personalized medicine. While recently developed melting probes have demonstrated significantly improved discrimination of mismatched (mutant) alleles from matched (wild-type) alleles, no effort has been made to design a simple melting probe that can reliably distinguish all four SNP alleles in a single experiment. Such a new probe could facilitate the discovery of rare genetic mutations at lower cost. Here we demonstrate that a melting probe embedded with a single locked thymidine monomer (t_L) can reliably differentiate the four SNP alleles by four distinct melting temperatures (termed the “4T_m probe”). This enhanced discriminatory power comes from the decreased melting temperature of the t_L -C mismatched hybrid as compared to that of the t-C mismatched hybrid, while the melting temperatures of the t_L -A, t_L -G and t_L -T hybrids are increased or remain unchanged as compared to those of their canonical counterparts. This phenomenon is observed not only in the HRM experiments but also in the molecular dynamics simulations.

INTRODUCTION

Genotyping of single-nucleotide polymorphisms (SNPs) is becoming a routine test in clinical laboratory as personalized medicine continues to develop.¹ Whereas many innovative SNP detection techniques have been developed in the past decades,^{2–6} high-resolution melting (HRM) measurement is probably the only method that is becoming a

*Corresponding Author. tim.yeh@austin.utexas.edu.

ASSOCIATED CONTENT

Supporting Information

Detailed preparation of probe-allele hybrids and measurements on high-resolution melting, circular dichroism, absorbance at 260 nm, fluorescence quenching, enthalpy and entropy. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

standard procedure in both research and clinical laboratories due to its homogeneous and closed-tube detection format.^{7–10} Since the first demonstration of melting curve analysis in conjunction with real-time PCR,^{11–13} HRM has been widely used for scanning of mutations in cancer-related genes^{14,15} and determining HIV diversity¹⁶ in clinical samples.

While HRM (based on FRET dyes¹¹ or saturating DNA dyes¹⁷) is a simple, rapid and inexpensive method for in-house SNP testing,¹⁸ a typical DNA melting probe (whether it is a binary probe,^{19,20} singly-labeled probe,^{21,22} unlabeled probe²³ and snapback primer²⁴) can only differentiate one fully matched allele from the other three single-mismatch containing alleles (hereafter denoted as mismatched alleles).^{20,25,26} Since the melting temperatures (T_m s) associated with the three mismatched hybrids (probe-allele hybrids) are often indistinguishable, additional approaches, such as sequencing, are required to differentiate these mismatched alleles (e.g. *CRP*,²⁷ *HNF1 β* ,²⁸ and *ABCB1*²⁹). There is no single melting probe that distinguishes the fully matched allele from the three mismatched alleles, and at the same time also differentiates among the three mismatched alleles. Although not all SNP homozygous variants are of clinical interest, we believe a melting probe with such an “ultimate discrimination power” is greatly beneficial as it facilitates the discovery of rare genetic mutations at lower cost.

To this end, our probe design consideration is different from that of other HRM researchers. Other researchers only aim to increase the T_m difference (T_m) between the fully matched hybrid and the three mismatched hybrids.²⁵ We, on the other hand, focus on increasing the T_m differences among the three mismatched hybrids themselves (while maintaining the T_m between matched and mismatched alleles). Our goal is to reliably identify each of the four SNP alleles with a specific T_m . We emphasize that we want to achieve such complete SNP differentiation in a single test tube using only one unlabeled melting temperature probe and a common DNA binding dye.

Here we demonstrate that a melting probe embedded with a single locked thymidine monomer (t_L) can reliably differentiate the four SNP alleles by four distinct melting temperatures (termed the “4 T_m probe”). This enhanced discriminatory power comes from the decreased melting temperature of the t_L -C mismatched hybrid as compared to that of the t-C mismatched hybrid, while the melting temperatures of the t_L -A, t_L -G and t_L -T hybrids are increased or remain unchanged as compared to those of their canonical counterparts. This phenomenon is observed not only in the HRM experiments but also in the molecular dynamics simulations. To our best knowledge, our t_L -containing probe is the first demonstration of a working 4 T_m probe.

RESULTS AND DISCUSSION

The thermal stability of a duplex containing a single centrally positioned mismatch (Figure 1) depends on the sequence and the type of mismatch. We termed the nucleotide on the probe that is right facing the SNP nucleotide the recognition nucleotide (RN). It has been previously shown that a DNA probe using thymidine as its RN (denoted as t-RN) could possibly give four distinct T_m s upon hybridization with the four SNP alleles.²⁰ However,

when testing this t-RN strategy on other DNA sequences, we noticed only two or three distinct T_m s could be resolved (Figures 2, S1 and S2).

Since the minimal T_m among the three mismatched alleles determines the specificity of the probe in distinguishing all four SNP alleles, we define this minimal T_m as the discriminatory power (DP) of the probe. As expected, the selection of RN strongly influences the DP of the probe (Figure 2). Using a *BRAF* mutation (oncogene mutation) as the model system (Tables S1 and S2),³⁰ when cytosine serves as the RN (c-RN), the discriminatory power of the *BRAF*c-RN probe is 0.2°C (Figure 2A, c-RN subplot; here DP is the T_m between the T- and A-allele melting curves). On the other hand, when thymidine serves as the RN (t-RN), the discriminatory power drops to 0.0°C (where the melting curves of the T and C alleles are nearly indistinguishable; $T_m < 0.1^\circ\text{C}$). Similarly, the DP of the a-RN probe is also 0.0°C. Guanine is the worst RN as the three associated mismatched melting curves are indistinguishable, mainly due to the fact that g·G, g·T and g·A mismatches have similar and relatively stronger stabilities as compared to other mismatches.³¹ Although in this *BRAF* model system the c-RN probe has a non-zero DP (0.2°C), c-RN is not a reliable 4 T_m probe design as other c-RN probes (which target distinct allele sequences) have shown a DP of 0.1°C or lower (Figure S1). We conclude that using canonical nucleotides as RNs, one cannot achieve reliable T_m discrimination among the three mismatched alleles while still staying with a simple single-RN melting probe design.

To improve discrimination among mismatched alleles, we turned to the locked nucleic acid (LNA) and tested the four LNA monomers (a_L , c_L , g_L and t_L) as the RN one-by-one. Although LNA has previously been incorporated into melting temperature probes to improve discrimination between match and mismatches,^{25,26} its capability in discrimination among mismatches has never been systematically evaluated. Here we carried out a complete set of experiment to compare DNA-RN and LNA-RN in SNP differentiation based on the same *BRAF* model (Figure 2). We found that locked thymidine monomer, when used as the RN (t_L -RN), can well resolve four distinct T_m s on the four alleles, with DP as high as 0.6°C (Figures 2A and 2B, t_L -RN subplots). Just by changing from t-RN to t_L -RN in the probe design, a net increase of 0.6°C in the discriminatory power (DP) is achieved. t_L also works well on other allele sequences, giving DP (from t-RN to t_L -RN) ranging from 0.5°C to 0.8°C (Figures S1 and S2).

In our standard HRM measurements, all hybrids were formed between a 60-nt long allele and a 48-nt long melting probe (equal molar; final concentration of the hybrid is 10 μM) (Figure 1). A commercial buffer, Precision Melt Supermix[®] from Bio-Rad which contains EvaGreen DNA dye, was used for the melting experiment. HRM measurements were carried out in a real-time thermal cycler (CFX Connect[™] from Bio-Rad, see SI Section I). While the best melting temperature discrimination is usually obtained with probe length around 20–30 nucleotides long,⁹ here we intentionally made our probes longer (48 nt) to highlight the superior DP offered by a single locked thymidine monomer modification. This is also to guarantee that the probe-allele hybrids have a B-form structure (Figure S3).³²

When the probe size becomes smaller, the free energy penalty resulting from a single mismatch becomes significant in the total free energy of binding,³³ thus increasing the T_m

between match and mismatches. Indeed, when a 25-nt long probe was used, the DP of the t-RN probe could be further increased to 1.4°C (Figure S4). We found blocking the 3' end of the probes by phosphorylation²³ does not affect the current T_m measurements as we do not perform temperature cycling (for PCR) in our experiments (Figure S5). The DP of the t_L-RN probe is not necessarily disappearing at high GC content (Figures 3 and S6). While the DPs of the t_L-RN probes (48-nt long) are relatively lower for the *SCA* (0.2°C, 60.4% GC content) and the *LFS* alleles (0.3°C, 64.6% GC content), the DP is still as high as 0.4°C for the *APOE* alleles³⁴ (75% GC content). Reducing the probe size from 48-nt to 25-nt long can certainly improve the DP of the t_L-RN probe at high GC content (Figure S4). Moreover, the t_L-RN probes are found working well in urea and fetal bovine serum solutions (with DP ranging from 0.4 to 0.8°C under various GC contents, Figure S7). Since only a limited number of allele sequences were tested in Figure 3, we could not rule out the possibility that the current t_L-RN probe design may fail on some particular target sequences. However, here we show that a simple t_L-RN probe design can achieve complete SNP differentiation (DP > 0.2°C) on a variety of allele sequences.

To understand whether or not the improved discrimination among mismatches is due to the use of DNA-binding dye EvaGreen, we repeated HRM measurements using a FAM-labeled probe²¹ and carried out a series of thermodynamic analysis based on absorbance at 260 nm (A_{260}).³⁵ In the FAM probe experiment, FAM was quenched by the two guanine bases in the allele upon hybridization, followed by fluorescence recovery upon melting²¹ (Figures S8 and S9). The FAM-based HRM measurements were carried out in the same real-time thermal cycler, but in a buffer without EvaGreen dye (SI Section I-A2). The changes of A_{260} upon temperature increase were used to calculate the melting temperatures (T_m s), free energies ($G_{37^\circ C}$), enthalpies (ΔH_h^0), and entropies (ΔS_h^0) for duplex dissociation (Table S3 and Figure S12). Two things are noticed when comparing the EvaGreen HRM results with the FAM and the A_{260} results. First, the t_L-induced DP enhancement is still observable in the two control experiments where no EvaGreen dye is used. The DP (of the t_L-RN probe) obtained from the FAM probe is almost identical to that from the EvaGreen dye (0.5°C vs. 0.6°C, Figure S8). Although the T_m values derived from the A_{260} curves are lower than those from the HRM measurements (Figure S13, presumably due to different buffer, hybrid size, and instrument used for the A_{260} measurement, SI Section I), the resulting T_m s (Table S3) not only well follow the trend observed in the HRM measurements (A>G>T>C alleles) but also become more differentiable when switching from the t-RN probe to the t_L-RN probe. Second, the DP improvement is quite specific to the t_L-RN probe. In the EvaGreen melting experiments, we often found c_L-RN probes lead to DP reduction (i.e. a negative DP, Figures 2 and S1). This is an interesting phenomenon – LNA modification at the probe's RN site definitely helps increase the T_m discrimination between matched allele and mismatched alleles, but it does not necessarily help improve the discrimination among mismatched alleles. In some cases, like the c_L-RN probes, discrimination among mismatches actually becomes worse. The A_{260} measurement results also confirm that the c_L-RN probe does not offer any benefit in discrimination among mismatches. Since absorbance measurements (Cary 50 Bio UV-Vis spectrometer from Varian) are not as sensitive and precise as HRM measurements (CFX Connect™ real-time system from Bio-Rad), a larger variation is seen in the T_m characterization using the A_{260} data ($\pm 0.3^\circ C$ based on A_{260} vs.

$\pm 0.1^{\circ}\text{C}$ based on HRM). As a result, the small DP reduction caused by the c-RN to c_L -RN swap (the negative DP seen in Figure 2) is not observed in the A_{260} measurements (Table S3).

Why only t_L -RN probes can reliably increase the DP but not other LNA probes? Why c_L -RN probes often give a negative DP? We found the answers to these questions lie in the fact that for hybrids containing certain mismatched pairs, T_m s are actually decreased when switching from DNA probes to corresponding LNA probes (Figure 4). As mentioned above, when LNA probes are used, the corresponding fully matched hybrids (i.e. a_L -T, c_L -G, g_L -C and t_L -A hybrids) always exhibit a significant increase in T_m (i.e. a positive T_m , red boxes in Figure 4). Substantial T_m increase is also seen when hybrids contain a t_L -G or g_L -T wobble pair. Whereas the T_m increase for the matched hybrids and the wobble hybrids (due to the use of LNA probes) is well understood, to our best knowledge no one has ever discussed the T_m response for the rest 10 mismatched pairs (a_L -A, c_L -C, g_L -G, t_L -T, a_L -C, c_L -A, a_L -G, g_L -A, c_L -T and t_L -C). Here we find that the T_m of the t_L -T hybrid is often nearly identical to or slightly higher than that of the t-T hybrid. Negligible or mixed T_m changes are observed for the g-A, g-G, c-A, c-T, a-A, a-C, and a-G hybrids after the switching (gray boxes in Figure 4). Interestingly, distinct from all other results, hybrids containing a c-C or t-C mismatch show consistently and substantially negative T_m s after the switching (blue boxes in Figure 4). Note that the T_m changes for the t-C and the c-T mismatches are not the same.

These unexpected lower T_m s of the c_L -C and t_L -C hybrids can explain the negative and positive DPs provided by the c_L -RN and t_L -RN probes, respectively. In the case of c-RN probe (Figure 2), the resulting c-C hybrid has the second highest T_m that can be specifically and substantially decreased when switching from the c-RN probe to the c_L -RN probe (i.e. melting curve shifted to the left). At the same time the melting curves of the c-T and c-A hybrids stay roughly where they are. As a result, the melting curves of the three mismatched hybrids become less differentiable after the switching (i.e. a negative DP). On the other hand, in the case of t-RN probe, the resulting t-G wobble hybrid has the second highest T_m that is significantly increased when switching from the t-RN probe to the t_L -RN probe (i.e. curve shifted to the right). As the curve of the t-A matched hybrid also shifts to the right after the t to t_L switching, the T_m difference between the t_L -G and t_L -A hybrids remains nearly unchanged. However, the T_m difference between the t_L -G wobble and the t_L -T mismatched hybrids is now larger than that of the corresponding canonical hybrids. This is because the T_m increase due to the t-G to t_L -G switch is always larger than the T_m increase due to the t-T to t_L -T switching. More importantly, the t_L -RN probe can now differentiate the originally indistinguishable melting curves of t-T and t-C hybrids, due to the fact that the T_m of the t-C hybrid is specifically and substantially decreased after the t to t_L switching (Figure 4). In summary, a t_L -RN probe tends to shift the t-A and the t-G melting curves to the right together and shift the t-C curve to the left, while it roughly maintains the position of the t-T curve, thus leading to four well-resolved melting curves for the four SNP alleles using only one melting probe (Figure 2). While the amount of right and left shift varies from one allele to another (i.e. variation of DP seen in Figure 3), we have found a simple melting probe design that can differentiate all four SNP variants by four distinct T_m s. We emphasize that Figure 4 aims to help readers to understand the effect of t_L on changing the T_m of matched

and mismatched pairs. The SNP discriminatory power of our probes should be read from Figures 2, 3, S1 and S2.

To further understand the molecular basis of these experimental findings, we employed molecular dynamics (MD) simulations to investigate the detailed melting processes given by the t-RN, t_L-RN and c_L-RN probes (Figures 5 and 6). In our simulations, the previously published AMBER99x force field^{36,37} was applied to the LNA residue (SI Section I-B). association fraction (AF), which is defined as the ratio between the number of stable base pairs and the total number of possible base pairs, was used to indicate duplex stability (AF of unity and zero represent full hybridization and complete melting, respectively; SI Section I-B6). A base pair was considered melted if the nitrogen-nitrogen distance between a complementary base pair (N1 for purines and N3 for pyrimidines) was greater than 5 Å. Initial and melted configurations of the t_L-C hybrid are shown in Figure 5A as an example (only the nearest neighbor bases around the mismatch are shown for clarity).

When comparing the simulation-derived AF with the experiment-obtained T_m, we can clearly see the correlation between them (Figures 5B and 5C). The benefit of the t_L-RN probe in SNP differentiation is confirmed by the broadened AF range observed in our MD simulations (Figure 5B). Although the resulting AF (change of AF due to the t-RN to t_L-RN switching) are found negative for the t_L-G and t_L-T hybrids (contrary to the T_m results shown in Figure 4), the duplex stability prediction follows the trend observed in the melting experiment. We emphasize that the goal of our simulation is to observe the broadened range of duplex stability upon the use of t_L-RN probe. We do not expect to match the AF with the T_m results shown in Figure 4. In our simulation, we can also clearly see that the c_L-RN probe is a much worse SNP probe when compared with the t_L-RN probe (Figure 5C). The AF of the c_L-A and c_L-T hybrids are indistinguishable, agreeing well with the indistinguishable c_L-A and c_L-T melting curves shown in Figure 2A.

MD trajectories can provide atomic insight and explanation for the two extreme cases that we have seen in the melting experiment: t_L-A vs. t-A hybrids and t_L-C vs. t-C hybrids. Both simulations and experiments suggest that t_L increases the duplex stability when paired with the SNP nucleotide A while t_L destabilizes the duplex when paired with C. Here we compute the hydrogen-bond (H-bond) probability from the MD trajectories (SI Section I-B9) for all nucleotides using distance and angle cutoffs of 3 Å and 20° (meaning any pair of nucleotides that meets the criteria is considered H-bonded). The H-bonds are calculated for all possible base pairs, and a 2D rainbow color probability map is generated for each duplex (Figure 6). Here an H-bond probability of unity indicates that the base pair is H-bonded throughout the analyzed trajectory while a probability of zero represents that no H-bonds are formed between the pair throughout the analyzed trajectory.

For the t_L-C hybrid the H-bond probabilities of the nearest neighbor pairs (pair #12 and #14 around SNP site, Figure 5A) are low (< 0.3), while for the t-C hybrid the nearest neighbor H-bond probabilities are noticeably higher (~ 0.4). As expected, the H-bond probabilities between the RNs and the SNP nucleotides (pair #13) are close to zero (< 0.1), indicating the lack of H-bonding at the center of the duplex due to mismatch (Figures 6A and 6B). In contrast to the mismatched hybrids, the nearest neighbor H-bond probabilities of the

matched t_L -A hybrid are higher (~ 0.5) than those of the t-A hybrid (~ 0.35) (Figures 6C and 6D). The H-bond probabilities at the center are ~ 0.45 for the t_L -A hybrid and ~ 0.4 for the t-A hybrid, indicating that H-bonding is also enhanced at the SNP site by the t_L -RN probe.

From these H-bond probabilities, a possible mechanism for the enhanced SNP discrimination by the t_L -RN probe can be identified. The chemical linkage in LNA limits the sugar puckering flexibility which in turn restrains the base mobility around χ torsion angle. As a result, the t_L -A pair is “locked” into the favorable (low energy) configurations, as shown by the enhanced H-bond probabilities at the center pair and the nearest neighbor pairs. Thus, there is a significant gain in the “binding” enthalpy. Correspondingly, the concept of pre-organizing ligand through covalent bonds to increase the protein-ligand binding free energy is a common practice in drug design, for entropic and/or enthalpic reasons.³⁸ Conversely, the t-C pair is a mismatch with very weak pairing interactions, and the LNA further restricts the ability of the bases to form energetically favorable interactions. This in turn allows for higher mobility of nucleotides around the t_L and leads to disruption of nearest neighbor H-bonds. While the weaker interactions could lead to higher entropy in the duplex, the loss in enthalpy is likely more dominant.

Overall, t_L is able to amplify the difference in stability between the t-A and the t-C hybrids. For c_L , the LNA also clearly increases the stability of the matched hybrid. Nonetheless, while “t” is able to form a wobble base pair with G, “c” essentially only forms an energetically favorable pair with G (Figure 4). All other SNP nucleotides paired with c are mismatches and the interactions are so weak that potential for discrimination among the mismatched SNPs (A, T and C) is completely lost. Therefore, the SNP discrimination power is encoded in the detailed chemistry and the restriction of sugar puckering by LNA, which subtly “shifts” the pairing strength and thus recognition thresholds.

CONCLUSIONS

We have demonstrated the use of a locked thymidine monomer in a melting probe design that can reliably differentiate all four SNP alleles by four distinct melting temperatures. We emphasize that such complete discrimination of homozygous SNP variants can be done in a single test tube using only one unlabeled melting temperature probe and a common DNA binding dye. Here we coin this new type of melting probe the “ $4T_m$ probe” – a melting probe that can exhibit four distinct melting temperatures upon hybridizing with the four SNP alleles. We emphasize that no melting probes at this moment can achieve the same SNP differentiation results as we have demonstrated in this report. Our findings are not only important to the HRM community but also important to the DNA origami^{39,40} and DNA-metal interaction communities^{5,30,41–45} as researchers in these two communities are always searching for new ways to fine tune the affinity between strands and fine tune the nucleobase environment around DNA-bound metal atoms.

While the t_L -RN probe is no doubt the first working $4T_m$ probe ever demonstrated, the current discriminatory power is still less than 1°C . This is because in this work we have not carried out any probe optimization (probe length is always 48-nt long with the RN right in the middle). Although a number of groups have demonstrated reliable SNP detection based

on small T_m differences,^{46–49} larger T_m differences ($> 1^\circ\text{C}$) will further improve SNP differentiation. With a variety of probe optimization strategies (such as shortening the probe length (Figure S4) and relocating the RN position in the probe), we expect to enhance DP above 1°C in the near future, thus facilitating the use of the $4T_m$ probe in clinical applications. While our t_L -RN probes can differentiate the heterozygous samples from the homozygous samples (Figure S10), we cannot discriminate among the six heterozygous samples (CG, CA, CT, GA, GT and AT) at this moment (Figure S11). Our future work will focus on the probe design that also enables differentiation among the six heterozygous samples by melting temperature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Ning Jenny Jiang for her assistance on melting analysis. This work is financially supported by Robert A. Welch Foundation (F-1833 to H.-C.Y.) and National Science Foundation (CHE-1611451 to H.-C.Y. and J.T.P.). PR and SYC are also grateful for the support by the Robert A. Welch Foundation (F-1691) and the National Institutes of Health (R01GM106137 and R01GM114237).

References

1. Ginsburg GS, McCarthy JJ. Trends Biotechnol. 2001; 19:491. [PubMed: 11711191]
2. Kwok PY. Annu. Rev. Genomics Hum. Genet. 2001; 2:235. [PubMed: 11701650]
3. Nakatani K. ChemBioChem. 2004; 5:1623. [PubMed: 15532027]
4. Syvänen A-C. Nat. Rev. Genet. 2001; 2:930. [PubMed: 11733746]
5. Yeh H-C, Sharma J, Shih Ie M, Vu DM, Martinez JS, Werner JH. J. Am. Chem. Soc. 2012; 134:11550. [PubMed: 22775452]
6. Yeh HC, Ho YP, Shih IM, Wang TH. Nucleic Acids Res. 2006; 34:e35. [PubMed: 16517937]
7. Knez K, Spasic D, Janssen KP, Lammertyn J. Analyst. 2014; 139:353. [PubMed: 24298558]
8. Audrezet MP, Dabricot A, Le Marechal C, Ferec C. J. Mol. Diagn. 2008; 10:424. [PubMed: 18687795]
9. Erali M, Voelkerding KV, Wittwer CT. Exp. Mol. Pathol. 2008; 85:50. [PubMed: 18502416]
10. Reed GH, Kent JO, Wittwer CT. Pharmacogenomics. 2007; 8:597. [PubMed: 17559349]
11. Lay MJ, Wittwer CT. Clin. Chem. 1997; 43:2262. [PubMed: 9439442]
12. Ririe KM, Rasmussen RP, Wittwer CT. Anal. Biochem. 1997; 245:154. [PubMed: 9056205]
13. Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ. BioTechniques. 1997; 22:176. [PubMed: 8994665]
14. Millward H, Samowitz W, Wittwer CT, Bernard PS. Clin. Chem. 2002; 48:1321. [PubMed: 12142390]
15. Hsieh LL, Er TK, Chen CC, Hsieh JS, Chang JG, Liu TC. Clin. Chim. Acta. 2012; 413:1605. [PubMed: 22579930]
16. Cousins MM, Ou SS, Wawer MJ, Munshaw S, Swan D, Magaret CA, Mullis CE, Serwadda D, Porcella SF, Gray RH, Quinn TC, Donnell D, Eshleman SH, Redd AD. J. Clin. Microbiol. 2012; 50:3054. [PubMed: 22785188]
17. Wittwer CT. Hum. Mutat. 2009; 30:857. [PubMed: 19479960]
18. Gundry CN, Dobrowolski SF, Martin YR, Robbins TC, Nay LM, Boyd N, Coyne T, Wall MD, Wittwer CT, Teng DHF. Nucleic Acids Res. 2008; 36:3401. [PubMed: 18448472]
19. Bernard PS, Pritham GH, Wittwer CT. Anal. Biochem. 1999; 273:221. [PubMed: 10469493]

20. Elenitoba-Johnson KSJ, Bohling SD, Wittwer CT, King TC. Nat. Med. 2001; 7:249. [PubMed: 11175859]
21. Crockett AO, Wittwer CT. Anal. Biochem. 2001; 290:89. [PubMed: 11180941]
22. Kurata S, Kanagawa T, Yamada K, Torimura M, Yokomaku T, Kamagata Y, Kurane R. Nucleic Acids Res. 2001; 29:e34. [PubMed: 11239011]
23. Zhou LM, Myers AN, Vandersteen JG, Wang L, Wittwer CT. Clin. Chem. 2004; 50:1328. [PubMed: 15166111]
24. Zhou LM, Errigo RJ, Lu HZ, Poritz MA, Seipp MT, Wittwer CT. Clin. Chem. 2008; 54:1648. [PubMed: 18676584]
25. Mouritzen P, Nielsen AT, Pfundheller HM, Choleva Y, Kongsbak L, Moller S. Expert Rev. Mol. Diagn. 2003; 3:27. [PubMed: 12528362]
26. Chou LS, Meadows C, Wittwer CT, Lyon E. BioTechniques. 2005; 39:644. [PubMed: 16312213]
27. Szalai AJ, Wu J, Lange EM, McCrory MA, Langefeld CD, Williams A, Zakharkin SO, George V, Allison DB, Cooper GS, Xie F, Fan Z, Edberg JC, Kimberly RP. J. Mol. Med. (Berl.). 2005; 83:440. [PubMed: 15778807]
28. Swen JJ, Baak-Pablo RF, Guchelaar HJ, van der Straaten T. Clin. Chem. Lab. Med. 2012; 50:279.
29. Fujii T, Ota M, Hori H, Sasayama D, Hattori K, Teraishi T, Yamamoto N, Hashikura M, Tatsumi M, Higuchi T, Kunugi H. J. Psychiatr. Res. 2012; 46:555. [PubMed: 22306099]
30. Yeh H-C, Sharma J, Han JJ, Martinez JS, Werner JH. Nano Lett. 2010; 10:3106. [PubMed: 20698624]
31. SantaLucia J, Hicks D. Annu. Rev. Biophys. Biomol. Struct. 2004; 33:415. [PubMed: 15139820]
32. Kypr J, Kejnovska I, Renciuik D, Vorlickova M. Nucleic Acids Res. 2009; 37:1713. [PubMed: 19190094]
33. Aboulela F, Koh D, Tinoco I, Martin FH. Nucleic Acids Res. 1985; 13:4811. [PubMed: 4022774]
34. Poulson MD, Wittwer CT. BioTechniques. 2007; 43:87. [PubMed: 17695257]
35. Mikulecky PJ, Feig AL. Biochemistry. 2006; 45:604. [PubMed: 16401089]
36. Yildirim I, Kierzek E, Kierzek R, Schatz GC. J. Phys. Chem. B. 2014; 118:14177. [PubMed: 25268896]
37. Condon DE, Yildirim I, Kennedy SD, Mort BC, Kierzek R, Turner DH. J. Phys. Chem. B. 2014; 118:1216. [PubMed: 24377321]
38. Shi Y, Zhu CZ, Martin SF, Ren PY. J Phys Chem B. 2012; 116:1716. [PubMed: 22214214]
39. Subramanian HKK, Chakraborty B, Sha R, Seeman NC. Nano Lett. 2011; 11:910. [PubMed: 21235216]
40. Sanderson K. Nature. 2010; 464:158. [PubMed: 20220817]
41. Obliosca JM, Liu C, Yeh H-C. Nanoscale. 2013; 5:8443. [PubMed: 23828021]
42. Obliosca JM, Babin MC, Liu C, Liu Y-L, Chen Y-A, Batson RA, Ganguly M, Petty JT, Yeh H-C. ACS Nano. 2014; 8:10150. [PubMed: 25299363]
43. Juul S, Obliosca JM, Liu C, Liu Y-L, Chen Y-A, Imphean DM, Knudsen BR, Ho Y-P, Leong KW, Yeh H-C. Nanoscale. 2015; 7:8332. [PubMed: 25901841]
44. Chen Y-A, Obliosca JM, Liu Y-L, Liu C, Gwozdz ML, Yeh H-C. J. Am. Chem. Soc. 2015; 137:10476. [PubMed: 26261877]
45. Petty JT, Story SP, Hsiang JC, Dickson RM. J. Phys. Chem. Lett. 2013; 4:1148. [PubMed: 23745165]
46. Vossen RHAM, Aten E, Roos A, den Dunnen JT. Hum. Mutat. 2009; 30:860. [PubMed: 19418555]
47. Hung CC, Lin SY, Lee CN, Cheng HY, Lin CY, Chang CH, Chiu HH, Yu CC, Lin SP, Cheng WF, Ho HN, Niu DM, Su YN. Anal. Biochem. 2009; 389:102. [PubMed: 19328768]
48. Chen WJ, Dong WJ, Lin XZ, Lin MT, Murong SX, Wu ZY, Wang N. BMC Med. Genet. 2009; 10:45. [PubMed: 19480685]
49. Zhan XH, Zha GC, Jiao JW, Yang LY, Zhan XF, Chen JT, Xie DD, Eyi UM, Matesa RA, Obono MMO, Ehapo CS, Wei EJ, Zheng YZ, Yang H, Lin M. Exp. Ther. Med. 2015; 9:469. [PubMed: 25574218]

Figure 1.

Single-nucleotide polymorphism (SNP) detection using a melting temperature probe containing a single LNA thymidine monomer (t_L). The probe-allele hybrid contains a single centrally positioned t_L ·C mismatched pair, thus denoted as the “ t_L ·C mismatched hybrid” or simply the “ t_L ·C hybrid”. In our design, the t_L is termed the “recognition nucleotide” (RN) while the C is termed the “SNP nucleotide”. When t_L is employed as the recognition nucleotide, the 48-nt long melting probe is denoted as the “ t_L -RN probe”. Similarly, the 60-nt long SNP allele is denoted as the “C allele”. In this report, the lower case letters represent the RNs while the upper case letters represent the SNP nucleotides. The subscript L represents an LNA monomer.

Figure 2.

Melting curves of the 4 DNA and the 4 LNA probes hybridized with the four *BRAF* alleles.

(A) Four normalized melting curves of the four resulting probe-allele hybrids are shown in each subplot, where either a canonical nucleotide or an LNA monomer is used as the recognition nucleotide. Melting temperature differences (T_m) between two neighboring curves are directly shown in the plot. Melting curves are not differentiable when T_m is 0.1 °C or lower. Here the discriminatory power (DP) is defined as the minimal melting temperature difference (T_m) among the four hybrids (marked by underlines). The change of discriminatory power (ΔDP) refers to the DP change upon switching from a DNA probe to its corresponding LNA probe. Among various probes tested, only the t_L-RN probe well differentiates the 4 *BRAF* alleles by 4 distinct melting temperatures. (B) Corresponding first derivative plots ($-dF/dT$) of the melting curves shown in (A). Error bars (represented by color ribbons) show standard deviations from three trials.

Figure 3. Discriminatory powers (DP) of t-RN and t_L-RN probes for eleven different alleles with various percentages of GC content. Clearly t_L-RN promotes melting temperature differentiation among the three mismatched hybrids in all eleven samples.

Figure 4.

Change of melting temperature (T_m) upon replacing the canonical recognition nucleotides with the LNA monomers. The red and blue boxes (showing the average and the min/max range) represent increased and decreased T_m after switching from DNA-RN to LNA-RN, while the gray boxes represent negligible or mixed T_m changes. The lower- and upper-case letters represent the recognition nucleotides (RN) and the SNP nucleotides, respectively. Raw data are obtained from four different alleles with various GC content (*BUB1*, *RS*, *BRAF*, and *SCA*).

Figure 5. Molecular dynamics (MD) simulations of twelve 25-bp long hybrids, each consisting of a *BRAF* allele and a melting probe. (A) 3D views of the mismatch pair and the nearest neighbor bases in the t_L -C hybrid at initial and melted states. Inset shows the bases and their identification numbers in the hybrid. (B) Correlations between the simulation-derived association fractions (AF, at 70 and 75 $^{\circ}\text{C}$) and the experiment-obtained melting temperatures (T_m) for the t-RN ($R^2 = 0.93$) and the t_L -RN ($R^2 = 0.97$) probes. (C) Correlations between AF (at 70 and 75 $^{\circ}\text{C}$) and T_m for the c_L -RN ($R^2 = 0.94$) and t_L -RN (R^2

= 0.97) probes. n represents the number of repeated 1–1.5 μ s MD simulations (replicates). Error bars are standard deviations from replicates.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6.

Hydrogen-bond (H-bond) probability maps of the (A) t_L -C, (B) t-C, (C) t_L -A and (D) t-A hybrids. Pair #13 is where the SNP site locates (Figure 5A). These maps clearly show how the t_L modification promotes local H-bond probability around the SNP nucleotide A but demotes H-bond probability around C, thus enhancing the SNP discrimination power.